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Cis and *trans* requirements for stable episomal maintenance of the BPV-1 replicator

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Papillomavirus genomes are maintained as multicopy nuclear plasmids in transformed cells. To address the mechanisms by which the viral DNA is stably propagated in the transformed cells, we have constructed a cell line CHO4.15 expressing constitutively the viral proteins E1 and E2, that are required for initiation of viral DNA replication. We show that these viral proteins are necessary and sufficient for stable extrachromosomal replication. Using the cell line CHO4.15, we have shown that the bovine papillomavirus-1 (BPV-1) minimal origin of replication (MO) is absolutely necessary, but is not sufficient for stable extrachromosomal replication of viral plasmids. By deletion and insertion analysis, we identified an additional element (minichromosome maintenance element, MME) in the upstream regulatory region of BPV-1 which assures stable replication of the MO-containing plasmids. This element is composed of multiple binding sites for the transcriptional activator E2. MME appears to function in the absence of replication but requires E1 and E2 proteins for activity. In contrast to, for example, Epstein–Barr virus oriP, stably maintained BPV-1 plasmids are not subject to once-per-cell cycle replication as determined by density labelling experiments. These results indicate that papillomavirus episomal replicators replicate independently of the chromosomal DNA of their hosts.

Keywords: DNA replication/minichromosome maintenance/papillomavirus/replication origin

Introduction

In lower organisms, such as prokaryotes and budding yeast, replication origins contain well-defined *cis* sequences called ‘replicators’, and interaction of these sequences with a specific initiator protein complex leads to the initiation of DNA synthesis in these cells (Jacob *et al.*, 1963; DePamphilis, 1993; Stillman, 1994 and references therein). Extrachromosomal replicators generally, in addition to their origin function, encode functions that assure equal distribution of replicated molecules between daughter cells at cell division. For prokaryotic plasmids these partitioning functions are well studied and can be provided by several different mechanisms in bacterial cells (Nordström, 1990). In higher organisms less is known about mechanisms for partitioning of extrachromosomal replicators. For artificial plasmids in yeast, chromosomal centromeres can provide this function. In metazoan cells, one well studied example of a stable extrachromosomal replicator exists: the latent origin oriP from Epstein–Barr virus (EBV). The maintenance function of EBV requires the viral replication factor EBNA-1 and a series of binding sites for EBNA-1 termed the family of repeats (FR). A model that has been suggested for the function of the EBNA-1/FR combination is that EBNA-1 bound to FR provides physical retention of the oriP plasmids in the cell nucleus (Krysan *et al.*, 1989).

Papillomaviruses are also capable of stable extrachromosomal replication. Infection and transformation of the cells by papillomaviruses follows single hit kinetics (Dvoretzky *et al.*, 1980). The current view divides the viral life cycle into three stages (Botchan *et al.*, 1986). First, following initial entry, the papillomaviral genome is amplified in the cell nucleus, i.e. viral DNA is synthesized faster than chromosomal DNA and the copy number is increased. The second stage represents maintenance of the viral DNA at a constant copy number, and the latent phase of the viral infection is established. During the third, vegetative, stage of the viral life cycle, viral DNA amplification is initiated again, late proteins are synthesized and viral particles are assembled.

The E1 and E2 proteins are the only viral factors required for initiation of papillomavirus DNA replication (Ustav and Stenlund, 1991; Ustav *et al.*, 1991; Yang *et al.*, 1991; Chiang *et al.*, 1992; Kuo *et al.*, 1994). A similar, if not identical, set of cellular replication factors and enzymes, in addition to viral initiator proteins, is utilized by SV40 (Tsurimoto *et al.*, 1990; Weinberg *et al.*, 1990) and bovine papillomavirus-1 (Müller *et al.*, 1994) at the origin of replication to initiate DNA synthesis. Analysis of the essential *cis* sequences shows that the BPV-1 minimal origin (MO) (Ustav *et al.*, 1993) resembles a typical eukaryotic origin of replication (DePamphilis, 1993) and it has been suggested that this similarity could also be extended to the mechanisms of replication of all papovaviruses (Nallaseth and DePamphilis, 1994; Bonne-Andrea *et al.*, 1995). However, the ability of the papillomaviruses to be stably maintained as plasmids distinguishes them from other papovaviruses. It has been known for >10 years that BPV-1 replicates in transformed cells as a multicopy nuclear plasmid, which can persist in the tissue culture cells over long periods of time (Law *et al.*, 1981). This indicates that papillomaviruses have efficient mechanisms for control of copy number and partitioning in the transformed cells.

Very little, if anything, is known about the involvement of viral factors, *cis*-acting sequences and cellular factors in long-term persistence of papillomaviruses. Here we present an extension of our previous work that dealt only

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with short-term replication and describe the development of a system that has allowed us to dissect the process of long-term persistence and to define requirements for viral factors and *cis* sequences for long-term extrachromosomal replication. We demonstrate that DNA replication from the MO *per se* is not sufficient for long-term persistence, but that in addition another function is required. This function, which appears to be dependent on E1 and E2, is provided by binding sites for E2 protein, when linked to the MO sequence. We believe that the E2 transcription factor functions in stable replication by participating in the segregation/partitioning process of the BPV-1 origin plasmids.

Results

Construction of cell lines expressing E1 and E2 proteins

The E1 and E2 proteins of BPV-1 are necessary and sufficient for initiation of DNA replication from the viral origin of replication, and expression of these two proteins from heterologous expression vectors allows replication of an MO in transient replication assays (Ustav and Stenlund, 1991; Ustav *et al.*, 1991). However, due to the lack of persistence of the transfected expression vectors, replication cannot be monitored for more than few days after transfection. To determine whether additional *trans*-acting factors or *cis*-acting elements are required for long-term maintenance of the viral DNA, continuous expression of these two factors has to be assured. We therefore constructed several cell lines constitutively expressing the E1 and E2 proteins. Expression of these proteins was directed from integrated constructs for E1 protein from a cytomegalovirus (CMV) promoter (cell line CHO212) and for E2 protein from the HSP70 promoter (cell line CHO49). In the cell line CHO4.15, which expresses both E1 and E2, the E1 protein was expressed from the SRα promoter and the E2 protein from the HSP70 promoter. Selection of the respective cell lines and amplification of the expression units of interest was achieved by utilizing the glutamine synthetase minigene from the pSVLGS.1 plasmid, according to the protocol described earlier (Bebbington and Hentschel, 1987). Expression of E1 and E2 was identified by immunoprecipitation using specific rabbit polyclonal sera (data not shown) and by *in vivo* replication assays as illustrated in Figure 1. The three cell lines and the parental CHO cells were transfected with the BPV-1 origin-containing plasmid pUC/Alu in combination with E1 and E2 expression vectors, as indicated in Figure 1. The cell line CHO4.15, which expresses both E1 and E2, supports replication of the origin plasmids in the absence of exogenous E1 and E2 (lanes 1 and 2). The E2-expressing cell line, CHO49, supports replication in the presence of an E1 expression vector (lanes 3 and 4), but fails to do so without exogenous E1 (lanes 5 and 6). The E1-expressing cell line, CHO212, supports replication only in the presence of an E2 expression vector (compare lanes 7 and 8 with lanes 9 and 10). In the parental CHO cell line, co-expression of both E1 and E2 is required for replication (lanes 11 and 12). No replication of pUC/Alu can be detected in the absence of E1 and E2 (lanes 13 and 14).

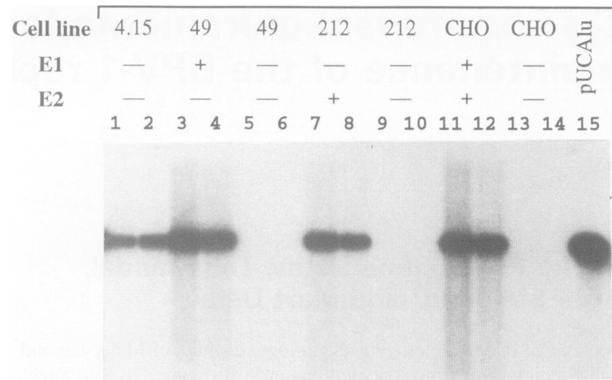


Fig. 1. Southern blot analysis of transient replication of the MO-containing plasmid (pUCAlu) in CHO4.15, CHO49, CHO212 and CHO cell lines. Transfection of pUC/Alu (50 ng) together with the indicated E1 or/and E2 expression vectors (250 ng) in the presence of carrier DNA (50 μg) was done as descibed in Materials and methods. At the indicated times (lanes 1, 3, 5, 7, 9, 11, 13: 48 h, and 2, 4, 6, 8, 10, 12, 14: 72 h), low molecular weight DNA was harvested and digested with the single-cutting enzyme *Hin*dIII and with *Dpn*I. Lanes 1 and 2: replication in the cell line CHO4.15; lanes 3 and 4: in the cell line CHO49 complemented with pCGEag; lanes 5 and 6: in the cell line CHO49; lanes 7 and 8: in the cell line CHO212 complemented with pCGE2; lanes 9 and 10: in the cell line CHO212; lanes 11 and 12: in the cell line CHO-K1 complemented with pCGEag and pCGE2; lanes 13 and 14: in the CHO K-1. pUC/Alu in lane 15 represents 50 pg of pUC/Alu marker linearized with *Hin*dIII.

E1 and E2 proteins are necessary and sufficient also for stable replication of the BPV-1 origin-containing plasmids; BPV-1 URR contains sequences required for long-term replication

To determine what viral *trans*-acting factors and *cis* sequences were required for long-term replication of BPV-1, we used the CHO4.15 cell line which constitutively expresses both the viral E1 and E2 proteins. Different fragments of BPV-1 were cloned into the vector pNeo5′ (Lusky and Botchan, 1984). This plasmid provides aminoglycoside 3′-phosphotransferase as a marker for selection of the cells in the presence of geneticine (G418). We used a 2.5 kb *Bgl*II fragment from the BPV-1 genome (nucleotides 6946–1515) as a starting fragment. This fragment contains the upstream regulatory region (URR) including the E2-dependent transcriptional enhancer, the MO and part of the early open reading frames (ORFs) (construct 12, Figure 2A). This plasmid, in parallel with an MO fragment in the same backbone (construct 13, Figure 2A), was transfected into the CHO4.15 cell line by electroporation and processed according to the scheme in Figure 3A. Some of the transfected cells were used after plating for analysis of transient replication. The rest of the cells were selected in the presence of G418 for 2 weeks, colonies were pooled or picked and grown under non-selective conditions for two additional weeks, at which time low molecular weight DNA was harvested and analysed for the presence of replicated plasmid. The ability of the origin-containing plasmids to replicate extrachromosomally in transient and long-term replication assays was examined by Southern analysis of the episomal DNA (see Materials and methods for details). The two plasmids containing the 2.5 kb *Bgl*II fragment and the MO respectively, replicated to comparable levels in the transient replication assay (Figure 3B). After selection in

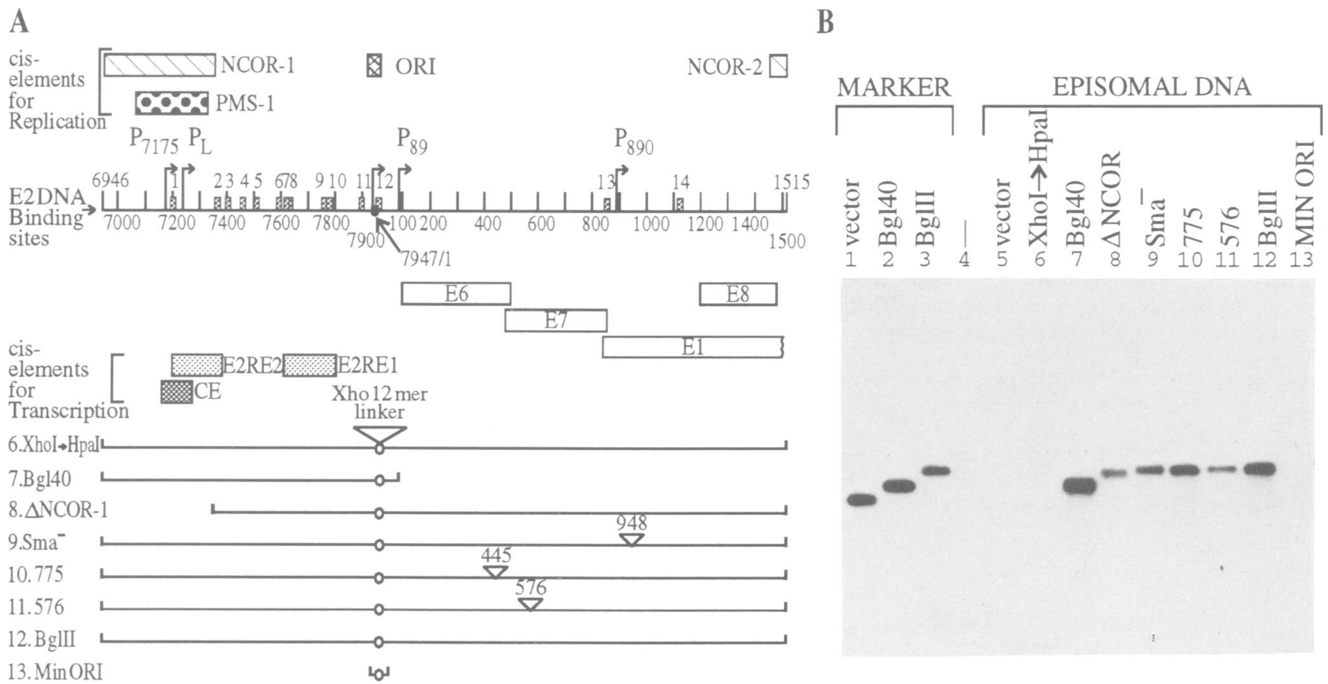


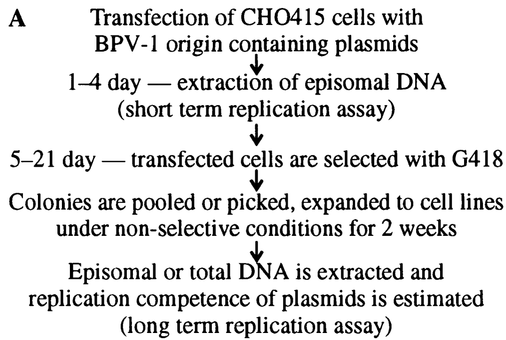
Fig. 2. Stable replication of the BPV-1 origin-containing plasmids in the CHO4.15 cell line. (**A**) Representation of the BPV-1 fragment (BPV nucleotides 6945–1515) used in this experiment. The respective mutants in this fragment are depicted and are described further in Materials and methods. The following genetic elements are indicated: NCOR-1 and 2, negative control of replication 1 and 2; PMS-1, plasmid maintenance sequence 1; ORI, minimal origin of replication; E2RE1 and E2RE2, E2-responsive enhancer 1 and 2 (Spalholz *et al.*, 1987); CE, constitutive enhancer (Vande Pol and Howley, 1992); E1, E6, E7 and E8, respective ORFs; P_{7175}, P_L, P_{89}, P_{890}, respective promoters; boxes indicate the location of 14 E2 binding sites in this fragment. (**B**) Southern blot analysis of stable cell lines for the presence of episomal plasmids. The marker lanes 1, 2 and 3 contain 100 pg of linearized vector pNeo5′, pNeoBgl40 and pNeoBglII, respectively. Low molecular weight DNA was extracted from CHO4.15 cells after transfection and G418 selection (see Materials and methods for details). The plasmids used were the vector plasmid pNeo5′ (lane 5), pNeoXhoI→HpaI with a disrupted E1 protein binding site (lane 6), pNeoBgl40 (lane 7), pNeoΔNCOR-I with a deleted NCOR (lane 8), mutants Sma⁻, 775 and 576 with a disrupted 5′ part of E1, E6 and E6/E7 ORFs respectively (lanes 9–11), the wild-type pNeoBglII (lane 12), and the MO-containing plasmid pNeoMO (lane 13).

the long-term replication assay, however, the result was very different. While the plasmid containing the 2.5 kb *Bgl*II fragment could be readily detected in episomal form, the MO-containing plasmid could not be recovered (compare lanes 12 and 13, Figure 2B). These results showed that sequences present in the larger plasmid, but absent in the plasmid containing only the MO, were required for long-term replication, and that an activity in addition to replication was required for stable maintenance of a plasmid in the CHO4.15 cells. These results also indicated that it was possible to maintain BPV-1 origin-containing plasmids for an extended period of time without the regulatory circuit that the viral genome can provide.

To determine what sequences within the *Bgl*II fragment were responsible for maintenance in the long-term assay, we generated mutations within that fragment and assayed these plasmids for maintenance. Initially, we generated mutations in the sequences suggested previously to have effects on replication. The BPV-1 *Bgl*II fragment contains coding sequences for three potential proteins, E6, E7 and the N-terminal part of the E1 protein. Mutations which interrupted E1 ORF, E6 ORF and E6/7 ORF (Schiller *et al.*, 1984; Lusky and Botchan, 1985; Berg *et al.*, 1986)—construct 9 (pNeo Sma⁻), construct 10 (pNeo775) and construct 11 (pNeo576), respectively—and, in addition, a deletion removing all coding sequences—construct 7 (pNeoBgl40), were introduced into pNeo5′ (Figure 2A). None of these mutations had a detectable effect on maintenance (compare lane 12 with lanes 7, 9, 10 and 11 Figure 2B), indicating that the coding sequences contained within the *Bgl*II fragment were dispensable. Consequently, E1 and E2 are the only viral gene products required for maintenance.

It has been suggested previously that BPV-1 URR contains two partially overlapping *cis*-regulatory control elements for stable replication, termed plasmid maintenance sequence 1 (PMS-1) (Lusky and Botchan, 1984) and negative control of replication 1 (NCOR-1) (Roberts and Weintraub, 1986). We deleted these two sequences by removing the sequence between the *Hin*dIII and *Mlu*I sites (nt 6959–7351, construct 8—pNeoΔNCOR, Figure 2A). This deletion had no deleterious effect on long-term replication of the plasmid (lane 8, Figure 2B), demonstrating that these putative elements were not required. Finally, as a negative control, an *Xho*I linker insertion mutant overlapping with the *Hpa*I site was generated (construct 6, Figure 2A). This mutation generated an origin defective for replication in the transient replication assay, and it is also defective for long-term replication (lane 6, Figure 2B).

We concluded from these results that *cis* elements required for stable replication of BPV-1 are located within the URR and are unrelated to the previously proposed elements PMS-1 and NCOR-1. We have named this *cis* element in the BPV-1 URR minichromosome maintenance element (MME).



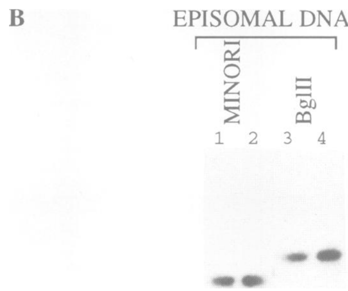


Fig. 3. (**A**) A scheme of the experimental protocol. (**B**) Short-term replication assay for the plasmids in the CHO4.15 cells. Low molecular weight DNA was extracted from the CHO4.15 cells transfected with the plasmids containing the MO or 2.5 kb *Bgl*II fragment and analysed by Southern blotting after digestion with *Dpn*I and linearizing enzyme *Xba*I (lanes 1 and 2) and *Hin*dIII (lanes 3 and 4). Lanes 1 and 2, episomally replicating MO-containing plasmid DNA extracted 36 and 60 h after transfection; lanes 3 and 4, episomal DNA extracted 36 and 60 h after transfection with the plasmid containing the 2.5 kb origin fragment.

MME is composed of redundant sequences

To define the sequences required for long-term replication, we generated a series of deletion mutants within the URR (Figure 4A). These deletions were made in the context of the plasmid pNeoBgl40 (BPV-1 sequence from 6946 to 63), and the deletion mutants were tested in the long-term replication assay. The first series of constructs (1–6, Figure 4A) had a fixed 5′ end (nucleotide 7187) and progressive deletions were made at the 3′ end of the URR (nucleotides 7892, 7834, 7771, 7475, 7389, respectively). The first four of these deletions (constructs 1–4, Figure 4A) were defective for stable replication (Figure 4B), but the plasmids with less extensive deletions (plasmids 5 and 6, Figure 3A) were maintained at the wild-type level (lane 5 and 6, Figure 3B). Another set of deletions (constructs 7–11, Figure 4A) had a fixed 3′ end (nucleotide 7890) and progressive deletions from the 5′ end (7476, 7611, 7673, 7771, 7834, respectively). Two mutants of this series were unable to replicate stably (deletions 7 and 8, Figure 4A and B), but plasmids with less extensive deletions replicated efficiently (lanes 9–11, Figure 4A and B). The results from the unidirectional deletions showed that a sequence in the vicinity of nucleotide 7600 was required for long-term replication (compare constructs 4 and 5, and 8 and 9). To map this sequence more precisely, we generated a third set of deletions. These deletions were constructed as scanning deletions (constructs, lanes 11–21, Figure 4A and B). Surprisingly, none of the introduced mutations resulted in loss of MME activity, demonstrating that no single unique sequence within the URR was required, but that maybe some redundant sequence element was responsible.

Binding sites for the E2 transcription activator comprise the MME

To address directly if the sequences in the vicinity of nucleotides 7600 were responsible for the MME activity, we generated a fragment between nucleotides 7590 and 7673, and inserted this fragment into the deletion mutant D221/234 (construct 1 in Figure 4A) to determine if replication in the long-term replication assay could be restored (this fragment corresponds to the sequence between the deletion end-points D134 and D11). This fragment inserted in three and six copies restored MME activity in the long-term replication assay (Figure 5, compare lanes 1 with 2 and 3). Known constituents of this fragment are three high affinity E2 binding sites. A possibility that occurred to us was that the MME activity was contributed by E2 binding sites. This would be consistent with the apparent redundancy, since the URR contains 10 binding sites for E2. To determine if E2 binding sites were involved in MME activity, we oligomerized high affinity E2 binding site 9 of the BPV-1 URR (5′-ACCGTTGCCGGT-3′) with six nucleotide spacing (Li *et al.*, 1989) and inserted these oligomers (10 copies) into the D221/234 deletion mutant. This insertion restored the MME activity (lane 4, Figure 5). To rule out involvement of other BPV sequences, we added 10 oligomerized E2 binding sites to the MO. Those constructs replicated with efficiency similar to plasmids with wild-type BPV-1 sequences in the stable assay (lane 5, Figure 5). However, plasmids with less than six additional oligomerized E2 binding sites failed to replicate in the long-term replication assay (data not shown). These results strongly suggest that binding sites for the E2 protein can be responsible for providing MME activity to the BPV-1 origin.

MME enhances the frequency of formation of G418-resistant colonies without replication

It has been observed previously that, for EBV, multimerized EBNA-1 binding sites (FR) are required for stable replication of oriP-containing plasmids in an EBNA-1-dependent fashion (Krysan *et al.*, 1989; Middleton and Sugden, 1994; Kirchmaier and Sugden, 1995). This activity can be measured by increased transformation frequency of the plasmids carrying FR, and is thought to be caused by enhanced nuclear retention of plasmids containing FR. To determine if a similar activity could be determined for MME, we measured the transformation frequency of four different plasmids. First, pNeo5′ carries the selectable neomycin resistance marker, but lacks BPV sequences and consequently is defective for replication in both the short- and long-term replication assays. Second, the MO plasmid, in addition, carries the BPV MO and is replication competent in the short-term replication assay but not in the long-term assay. The third plasmid pNeoXhoI→HpaI carries the whole *Bgl*II fragment, and is thus nominally capable of maintenance, but because of the linker insertion in the E1 binding site the plasmid is defective for replication. The fourth plasmid pNeoBgl40 is replication com-

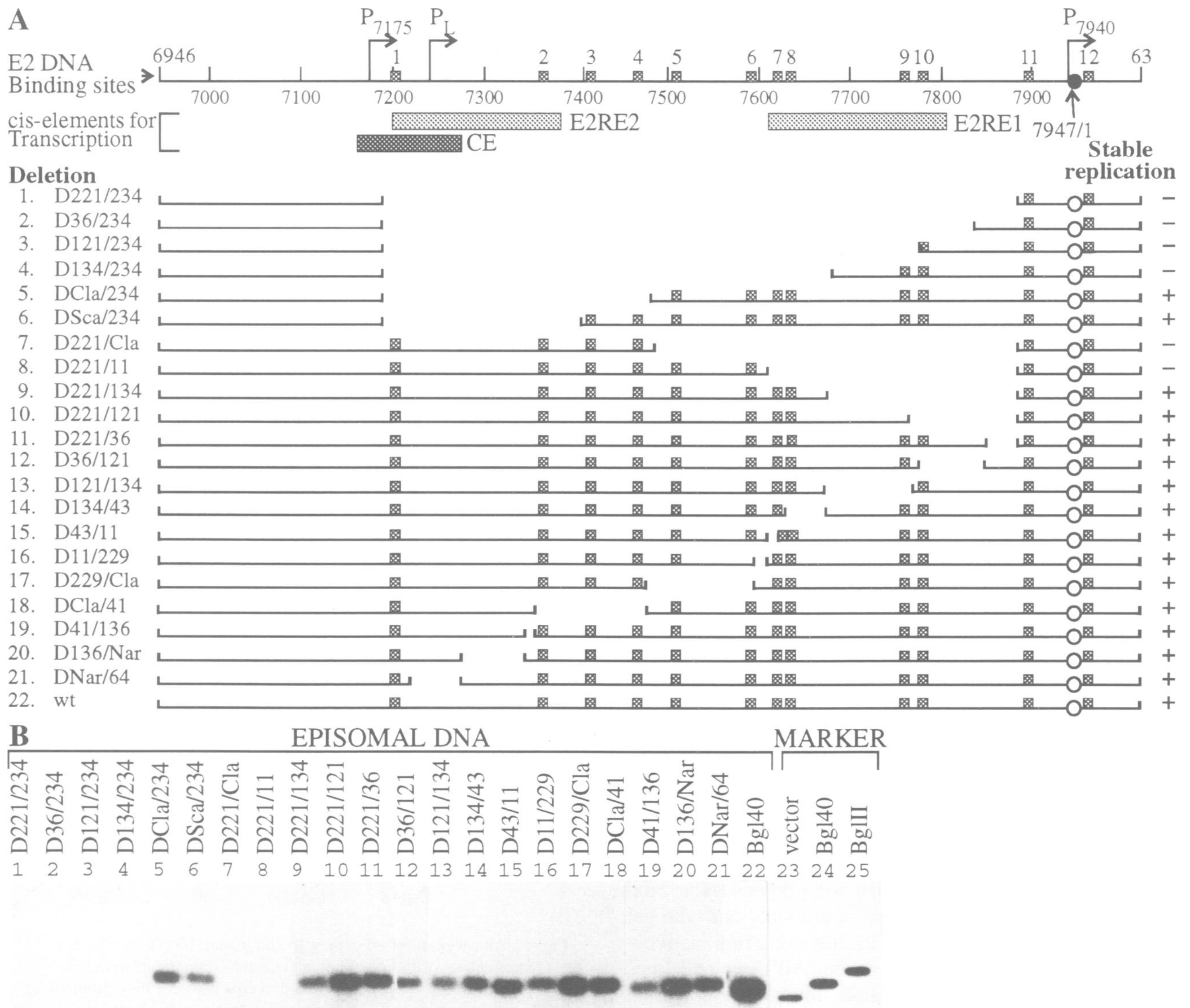


Fig. 4. Stable extrachromosomal replication of the plasmids with deletions in the URR in CHO4.15 cells. (**A**) Representation of BPV-1 fragments with respective deletions. P_{7175}, P_L and P_{7940}, the respective promoters in this fragment; E2RE1 and E2RE2, the E2-responsive enhancers; CE, constitutive enhancer; boxes indicate the E2 protein binding sites. End points of the respective deletions are given in Materials and methods. The circle indicates the location of the MO. The ability of respective deletion mutants to function in a long-term replication assay is indicated by (+) or (−). (**B**) Low molecular weight DNA was extracted from the cells, transfected with the respective plasmids and selected for G418, digested with the linearizing enzyme *Hin*dIII and analysed by Southern blot (lanes 1–22). Lanes 23, 24 and 25 contain 100 pg of linearized pNeo5′ vector, pNeoBgl40 and pNeoBglII marker DNA.

petent in both the short- and long-term replication assays. We used CHO4.15 cells to measure the transformation frequency of these different plasmids (Figure 6). The vector with the selectable marker only and the plasmid containing the MO transformed CHO4.15 cells with similar frequency (14 and 16 colonies/10^5 cells, respectively). In the parallel experiment, pNeoXhoI→HpaI, which is replication defective with a mutant E1 binding site but carries the sequences required for MME activity, transformed CHO4.15 cells 4–5 times more efficiently than vector alone or a plasmid with the MO (68.6 colonies/10^5 cells) (Figure 6A). The plasmids with the complete origin transformed cells with ~100 times higher efficiency than the vector containing only the MO (~1600 colonies/10^5 cells). These results indicated that MME activity could be measured in a stable transformation assay even in the absence of replication. When the same experiments were also performed in CHO212 (E1 cell line) and CHO49 (E2 cell line), all plasmids transformed with approximately the same efficiency in these two cell lines (Figure 6B and C). We conclude that enhanced transformation activity requires both E1 and E2 proteins.

We measured the possible effect of the MME (or oligomerized E2 binding sites) on plasmid retention in the short-term assay, as was done with the EBV oriP-containing plasmids. However, attempts to reproduce a direct effect on nuclear retention in the transient assay failed to show a significant effect (data not shown).

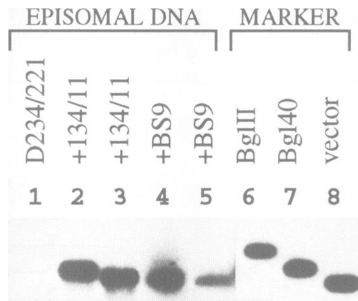


Fig. 5. Restoration of stable replication of the plasmids by the oligomerized E2 binding sites. Low molecular weight DNA was extracted from the G418-resistant cells and linearized with *Hin*dIII (lanes 1–4) or with *Xba*I (lane 5). Lane 1 represents analysis of the DNA from the cells transfected with the original D234/221 mutant with a deleted MME. Insertion mutants with E2RE1 (BPV nucleotides 7611–7673) cloned into D234/221 adding back 18 and nine E2 binding sites restored stable replication of the plasmid (lanes 2 and 3). A mutant with 10 oligomerized E2 binding sites cloned into D234/221 (lane 4) and a mutant with a deleted PMS1 with 10 oligomerized E2 binding sites inserted (lane 5) replicate in the long-term assay. Lanes 6–8 contain 100 ng of the respective linearized marker DNA.

BPV origin plasmids replicate at ~15 copies per haploid genome

One of the factors that is expected to influence the stable maintenance of a plasmid is the copy number. We therefore performed experiments to estimate the average number of episomal copies per haploid genome in different established cell lines. After digestion with the single-cut restriction endonuclease *Hin*dIII, total DNA from three independent cell lines—pNeoBgl40, pNeo41/Cla and pNeoSca/234—was loaded in equal amounts onto the gel and was analysed by Southern blotting using radioactively labelled BPV-1 origin and *neo* probe. All three cell lines contained approximately the same number of episomal plasmids; 15 copies per haploid genome (Figure 7A). Even though no specific effort was made to determine the number of integrated copies, digestion with non-cutter enzymes did not change the appearance of the three forms and oligomers of the plasmid (Figure 7B). Consistent with previous reports, the majority of the plasmids was present in the oligomeric form. We conclude from these results that the plasmids are mostly episomal in the CHO4.15 cell line under the conditions used.

Mode of replication

One explanation for the apparent high stability of BPV-1 plasmids in the cells could be that they are subject to the cellular once-per-cell cycle replication control. To determine if this was the case, we performed density labelling experiments using the cell line CHO4.15 containing the replicating plasmid pNeoBgl40. The experiments were performed by continuous labelling of the cells with 5′-bromodeoxyuridine(BrdU) for 3.5, 9.5, 15 or 24 h. Low molecular weight DNA and total chromosomal DNA were extracted after each time point and analysed by CsCl gradient centrifugation, followed by slot blotting, and hybridization with plasmid probe or genomic DNA probe to identify the peaks in the gradient. The density gradient profiles are shown in Figure 8. The data is summarized

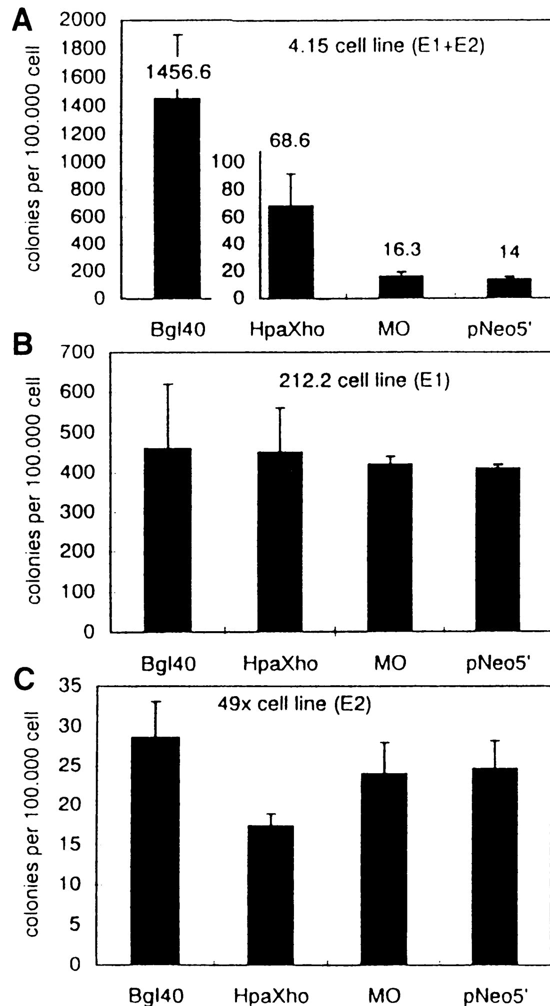


Fig. 6. Transformation efficiency (colonies per 100 000 cells) for G418 resistance of the plasmids pNeoBgl40 (Bgl40), pNeoXhoI→HpaI (HpaXho), pNeoMO (MO) and vector pNeo5′. Five hundred ng of the respective plasmid DNA were electroporated into the cells, and 84 h later the cells were trypsinized, counted and plated. Complete medium containing 450 μg/ml of G418 was used for selection. Colonies were counted on the 10th day of selection. The values presented are the average of three independent measurements. (**A**) CHO4.15 cells, (**B**) CHO212 cells and (**C**) CHO49 cells.

in the table as fractions of Bgl40 DNA and CHO chromosomal DNA that had incorporated no BrdU (light–light, LL), BrdU incorporated into one strand (heavy–light, HL) or into both strands (heavy–heavy, HH) in the CHO4.15 cells stably transformed by this plasmid (Figure 8). After labelling with BrdU for 3.5 h (Figure 8a and e), the episomal BPV-1 origin-containing plasmids are distributed between three forms of DNA: 5% heavy–heavy, 19% heavy–light and 76% light–light, while chromosomal DNA is distributed between two forms: 27% heavy–light and 73% light–light. After labelling for 9.5 h, the plasmid has accumulated a considerable amount (21%) of heavy–heavy DNA, while chromosomal DNA shows no detectable signal in the heavy–heavy area. After labelling for 15 h, distribution of the episomal DNA is 34% heavy–heavy, 38% heavy–light and 28% light–light. At the same time chromosomal DNA shows still very little, if any, heavy–heavy DNA. After labelling for 24 h, episomal DNA was preferentially in the heavy–heavy fraction of

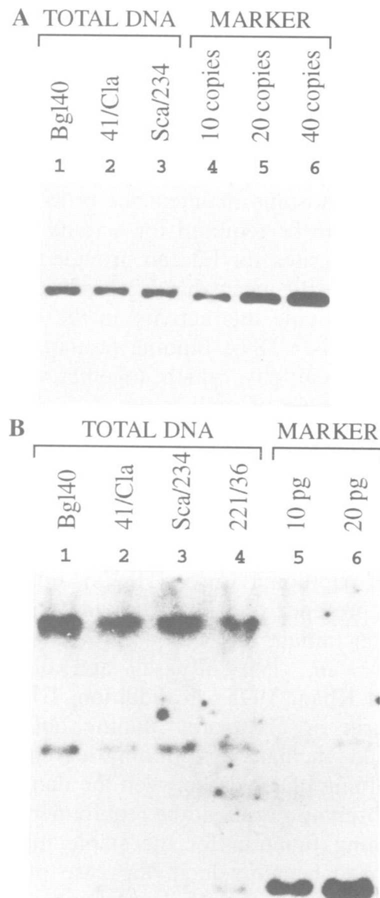
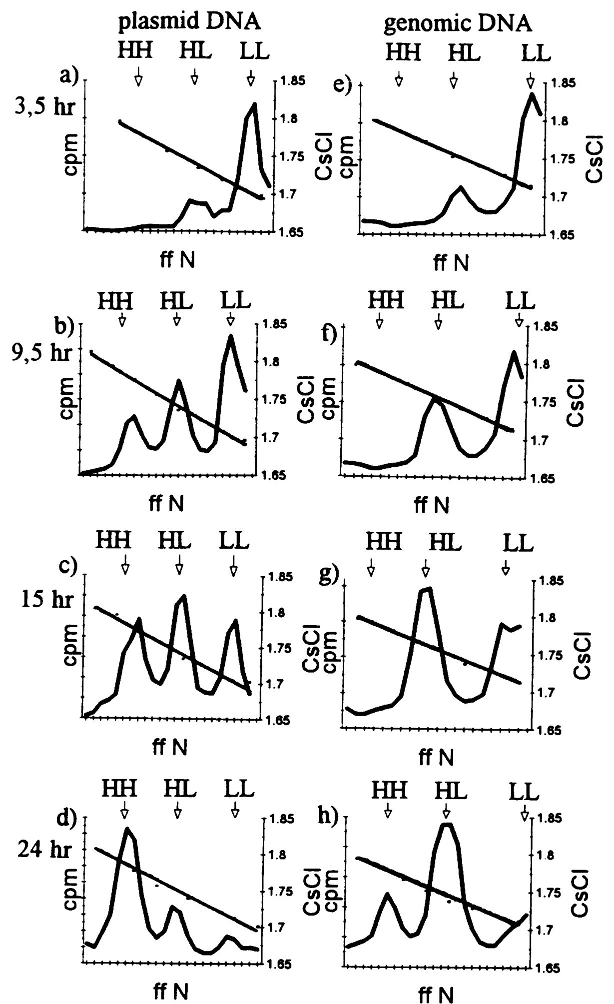


Fig. 7. Analysis of the state and copy number of BPV-1 origin-containing plasmids in CHO4.15 cells. (**A**) Copy number measurement of BPV plasmids stably replicating in CHO4.15 cells. Total DNA was extracted from the stable cell lines and subjected to linearization with *Hin*dIII. Lanes 1–3 represent analysis of 2 μg of total DNA from three independent cell lines; a series of plasmid dilutions for copy number reconstruction is included in lanes 4–6. (**B**) State of BPV plasmids. Total DNA (2 μg) from four independent cell lines cut with plasmid non-cutter *Apa*I is analysed (lanes 1–4). The respective markers of uncut plasmid DNA are shown in lanes 5 and 6.



	plasmid DNA			genomic DNA		
	HH	HL	LL	HH	HL	LL
3,5 hr	5%	19%	76%		27%	73%
9,5 hr	21%	34%	45%		44%	56%
15 hr	34%	38%	28%		58%	42%
24 hr	66%	24%	10%	24%	59%	17%

Fig. 8. Comparison of the stable replication modes of BPV origin-containing plasmid and chromosomal DNA in the CHO4.15 cells. BrdU labelling of the CHO4.15 cells carrying stably replicating pNeoBgl40 was done for (**a**) and (**e**) at 3.5 h, (**b**) and (**f**) at 9.5 h, (**c**) and (**g**) at 15.0 h and (**d**) and (**h**) at 24 h. Episomal (a–d) and total chromosomal DNA (e–h) were prepared at the respective time points and analysed as described in Materials and methods. CsCl gradients were aliquoted, denatured, neutralized and loaded onto the nylon filters by slot-blotter and hybridized with radioactive BPV-1 origin probe for episomal DNA and with radioactive total CHO DNA for genomic DNA gradients. The intensity of hybridization was quantitated by use of the PhosphorImager.

the DNA (66%), with 24% heavy–light and 10% light–light, while chromosomal DNA shows a considerable amount of heavy–heavy DNA (24%), but with most of the DNA still in the once replicated DNA fraction. These results are consistent with a doubling time of ~16 h for the pNeoBgl40-containing cell lines. The considerable percentage of unreplicated chromosomal DNA after 24 h is likely to be due to growth arrest of a fraction of the cells by the conditions used for BrdU labelling. It appears clear from these results that the stably maintained pNeoBgl40 plasmid does not replicate once per cell cycle, and stable maintenance of the BPV-1 plasmids is not a function of once-per-cell cycle replication control.

Discussion

Requirements for stable replication

We describe here the development of a system to study an aspect of papillomavirus replication that previously has not been addressed. We have developed this system to dissect papillomavirus replication and to uncouple viral gene expression from DNA replication. The behaviour of BPV-1 origin-containing plasmids in this stable replication system is very similar to the behaviour of the viral genome in transformed mouse cells in several important aspects. The copy number of the stably replicating plasmids is ~15 copies per haploid genome; CHO cells are diploid, and the cells therefore contain ~30 copies per cell. This copy number is of similar magnitude to the 50 to several hundred copies that has been estimated for the BPV-transformed cell line ID13. The stability of these plasmids

also appears to be very high. Even after passage of these cell lines in the absence of selection for 8 weeks, no loss of plasmid could be detected (data not shown).

This system has enabled us to resolve some long standing questions concerning the requirement for viral gene products and *cis*-acting elements for long-term replication. Earlier results have suggested that several viral proteins and also *cis*-acting elements are involved in stable replication (Lusky and Botchan, 1985, 1986; Berg *et al.*, 1986), although other laboratories have not been able to confirm these results (Jareborg *et al.*, 1992; Hubert and Lambert, 1993). We have previously demonstrated that none of these had an effect on replication in transient, short-term replication assays. In this study, we have reproduced long-term replication under well defined conditions and we show that these proposed elements and viral factors also have no detectable activity in the long-term replication assay. Thus, the E6 and E7 ORFs, the N-terminal part of E1, and the NCOR and PMS sequences appear to have no direct effect on replication. Since the earlier work was done in the context of the whole BPV-1 genome, the observed effects of some of the mutations (Berg *et al.*, 1986) may have been caused by effects on the expression of the replication factors E1 and E2. Instead, we have identified a new activity that, in combination with replication, is specifically required for long-term replication. We have termed this activity minichromosome maintenance, and we have demonstrated that this activity is provided by multimerized E2 binding sites.

The mechanism of action of the MME is unclear at present, but three main modes can be envisaged. First, the MME could affect efficiency of initiation of replication. Although no difference in replication initiation can be detected during the time course of the transient replication assay (Ustav *et al.*, 1991), it is conceivable that a gradual accumulation of methylated residues at the origin of replication or some other form of modification (including nucleosome occlusion) could prevent initiation of replication and result in gradual loss of replication activity. It is possible that the MME can affect these processes and prevent inactivation of the origin. Alternatively, the MO-containing plasmids are competent for overreplication during the S phase of the cell cycle, which could be toxic to cells. The MME could prevent this process, in analogy to the function of iterons for certain bacterial plasmids (for review, see Nordström, 1991). Third, the MME could influence the partitioning process, thereby affecting the loss rate of plasmids during cell division. At present, we have no direct information to distinguish between these models. However, the experiments measuring the transformation frequency can give some hints. The observation that an increase in transformation frequency can be detected even in the absence of replication is an indication that MME can act independently of replication, which would rule out the first two models. While our experiments do not prove conclusively that the increased transformation frequency that we can measure for non-replicating plasmids is the same activity that is required for long-term replication, similar effects on transformation frequency have been observed for the FR in oriP of EBV, and we believe that these two activities are related. The absence of MME activity in the E2 cell line CHO49 and in the E1 cell line CHO212 makes it unlikely that increased transformation frequency is an effect of activation of transcription of the integrated selection marker in the CHO4.15 cells. It has been shown previously that E1 and E2 proteins are able to form a stable complex in solution (Mohr *et al.*, 1990; Blitz and Laimins, 1991). It is reasonable to assume that this interaction is also involved in the minichromosome maintenance process, since both proteins appear to be required for activity. The fact that multiple binding sites for E2 can provide MME activity does not necessarily mean that E2 binding sites and E2 protein alone provide this activity in the context of the viral origin. E1 is a DNA binding protein, albeit with a low sequence specificity, which, together with E2, binds to sites at the origin of replication. If, as in the case of replication, the primary function of E2 is to confer sequence specificity to E1, E1 may be the active component required for MME activity but the presence of E2 and E2 binding sites may be required for E1 to bind to the plasmid and exert its function. It is clear that for replication of some human papillomaviruses (HPVs), E2 binding sites alone in the presence of E2 can provide a platform from which E1 can initiate replication in transient replication assays (Lu *et al.*, 1993; Russell and Botchan, 1995; Svedrup and Khan, 1995). In addition, E1 may have a nuclear matrix or chromatin binding ability, and this activity could mediate partitioning/segregation of the origin-containing plasmids between the daughter cells.

Similar observations about the requirement for segregation/partitioning function for the stable maintenance of the origin have been made in the case of autonomous replication sequence (ARS)-containing plasmids of budding yeast (Struhl *et al.*, 1979) and EBV oriP-containing plasmids (Yates *et al.*, 1985). Replication from oriP requires *cis*-acting elements (FR and the dyad symmetry element) and the viral origin binding protein, EBNA-1 (Yates *et al.*, 1984, 1985). FR has an effect on the stable extrachromosomal replication of the oriP by nuclear retention of the FR-containing plasmids in mitosis. This activity directs plasmids into the newly forming nucleus in the telophase stage of cell division (Krysan *et al.*, 1989). We cannot exclude the possibility that the MME of the papillomaviruses and E1 and E2 proteins act in the similar way.

It is interesting to note that the ability of the MO-containing plasmids to replicate appears to have no detectable effect on transformation frequency. One might have expected that an increase in the quantity of plasmid DNA in the cells as a result of replication would lead to a higher frequency of integration. However, this appears not to be the case, possibly because these minichromosomes are poor substrates for the required recombination events or are lost with very high frequency at cell division. The very large increase in transformation frequency of the plasmids with both an MO and an MME compared with integrating marker presumably reflects the fact that the two functions together can bypass the requirement for integration, possibly by providing an efficient segregation/partitioning function in addition to replication.

We have shown that, in the case of BPV-1, the MME activity can be provided by binding sites for E2 protein. Other papillomaviruses, especially HPVs, contain less than four of these elements. It has been shown that a number of HPVs (HPV16, 18, 31, 33, 52) could be

associated with cervical carcinoma (reviewed in DeVilliers, 1989) and are frequently found to be integrated in the tumour tissue. Our data would indicate that this could be caused by insufficient activity of the MME for stable extrachromosomal maintenance, which leads to accidental integration of the viral genome, deregulation of the expression of the viral oncogenes E6 and E7 and, following accumulating changes, would lead to the fully transformed tissue. We are working currently to identify and characterize the MME within HPVs.

Amplification and subsequent overexpression of oncogenes occur in many human and rodent tumour cells *in vivo*. Initial molecular intermediates of gene amplification result in generation and subsequent oligomerization of circular precursors, which eventually give rise to the double minute chromosomes (DMs) lacking centromeres (Windle *et al.*, 1991; Windle and Wahl, 1992). Since double minute chromosomes contain actively transcribed genes (Wahl, 1989), it is possible that cellular transcription factors and their binding sites at DMs provide MME activity for DMs and that way ensure stable maintenance of the DMs in the tumour cells—the same way as E1 and E2 proteins with respective E2 binding sites provide that function to the BPV-1 origin plasmids.

Mode of replication

In eukaryotes, the entire genome is replicated precisely once in each cell cycle. Using BPV-1-transformed C127 cells, contradictory results about whether replication of BPV-1 is regulated in the same fashion as chromosomal DNA have been reported. First, it has been shown that the density labelling pattern resulting from continuous labelling of the BPV-1 DNA in a non-synchronous culture of the cell line ID13 parallels that of chromosomal DNA, and it has been concluded that BPV-1 also replicates once per cell cycle (Botchan *et al.*, 1986). However, using ID13 and two additional BPV-1-transformed cell lines, other studies (Gilbert and Cohen, 1987; Ravnan *et al.*, 1992) found by pulse-labelling and density gradient analysis that BPV-1 DNA replicates in transformed mouse fibroblasts by a random-choice replication mechanism. Our results show that E1- and E2-dependent stable replication of the BPV-1 origin is consistent with a random mode of replication, and consequently that once-per-cell cycle replication is not a prerequisite for stable replication. These data indicate that BPV-1 plasmids utilize a completely different set of control mechanisms compared with EBV, which replicates strictly once-per-cell cycle (Yates and Guan, 1991). In contrast to EBV, BPV-1 origin plasmids behave more like bacterial plasmids and are uncoupled from the strict control of initiation of host DNA synthesis. These results demonstrate that, in mammalian cells as well as in prokaryotic systems, different strategies can be utilized to achieve the same net result, i.e. stable maintenance of extrachromosomal elements. There is no indication that BPV-1 (as well as all other papillomaviruses) carries more than one replication origin. It means that the same origin is used at the initial stage of the amplification, in the maintenance and possibly at the late stage of the viral life cycle.

Materials and methods

Plasmid constructions

Expression vectors. The E1 and E2 protein expression vectors pHSE2 (Szymanski and Stenlund, 1991), pCGE2 and pCGEag (Ustav and Stenlund, 1991) have been described earlier. The E1 expression vector pE1-1×5 contains the BPV-1 E1 ORF with *Xho*I linkers (within nucleotides 619–2757) and carries a point mutation at the splice donor site at nucleotide 1235. This fragment was cloned into the *Xho*I site downstream of the SRα promoter in plasmid pBJ5GS (kind gift from Dr L.Berg).

Origin plasmids. All origin fragments of the BPV-1 genome were cloned in the sense orientation into the *Bam*HI site of pNeo5′ (Lusky and Botchan, 1984). pNeoBglII contains a *Bgl*II fragment from BPV-1 (nt 6946–1515) cloned in the sense orientation relative to the transcription of the *neo* gene. pNeoXhoI→HpaI contains the same *Bgl*II fragment with an *Xho*I linker insertion in the *Hpa*I site (Ustav *et al.*, 1991). pNeo576, pNeo775 and pNeoSma⁻ plasmids contain the same *Bgl*II fragment with the mutations 576, 775 and Sma⁻ which affect E6/7 ORF, E6 ORF and the 5′ part of the E1 ORF, respectively (Schiller *et al.*, 1984; Lusky and Botchan, 1985; Berg *et al.*, 1986). pNeoΔNCOR has a deletion between *Hin*dIII (nt 6958) and *Mlu*I (nt7351). pNeoBgl40 contained a BPV-1 fragment from nucleotide 6946 to 63, which was amplified by PCR using the respective primers and cloned in the sense orientation into the *Bam*HI site. pNeoMO contained the MO sequence (nucleotides 7914–27) cloned into the *Bam*HI site. Linker deletion mutants of the BPV-1 genome (Szymanski and Stenlund, 1991) were used as templates for PCR. Primers [5′-AAAAGCTTTCTTTGGACTTAGA-3′ (BPV-1 nucleotides 6959–6979) and 5′-ATAGCCAGCTAACTATAGATCT-3′ (BPV-1 nucleotides 45–63 flanked by the *Bgl*II site)] were used to amplify origin fragments. PCR products were cloned into the *Hin*dIII and *Bam*HI site of pNeoBgl40. Deletion mutants lacked the following sequences: D221/234, 7187/7892; D36/234, 7187/7834; D121/234, 7187/7771; D134/234, 7187/7673; DCla/234, 7187/7475; DSca/234, 7187/7389; D221/Cla, 7476/7892; D221/11, 7611/7892; D221/134, 7673/7892; D221/121, 7771/7892; D221/36, 7834/7892; D36/121, 7771/7834; D121/134, 7673/7771; D134/43, 7622–7673; D43/11, 7611/7622; D11/229, 7597/7611; D229/Cla, 7476/7597; DCla/41, 7355/7476; D41/136, 7344/7356; D136/Nar, 7273/7344; DNar/64, 7214/7273; and junctions contained 8mer *Bam*HI linkers. D221/234+134/11×3 contains an insertion of three copies of the fragment 7590–7673 and D221/234+134/11×6 an insertion of six copies. D221/234+10BS9 has an insertion of 10 copies of the E2 protein binding site 9. DHindIII/221+10BS9 is a deletion between nucleotides 6959 and 7892 which carries 10 copies of oligomerized E2 binding site 9. All deletion and insertion mutants were verified by sequencing.

Construction of cell lines

CHO-K1 (Chinese hamster ovary, ATCC CCL 61) was used as the parental cell line to express BPV-1 replication proteins.

E2 cell line CHO49. The E2 expression vector pHSE2 was linearized with *Xho*I and the plasmid pBJ5GS carrying the glutamine synthetase minigene expression unit (Bebbington and Hentschel, 1987) (kind gift of Dr L.Berg) was linearized with *Sal*I endonuclease. The plasmids were mixed in a 1:1 ratio and ligated into the concatemers at high DNA concentration (300 μg/ml) overnight at 16°C using T4 DNA ligase.

Ten micrograms of the ligated DNA were mixed with 50 μg of carrier DNA and was electroporated into 7×10^6 CHO-K1 cells using Ham's F12 medium supplemented with 10% fetal bovine serum (FBS) at 220 V, using a Bio-Rad electroporation apparatus at the capacitance setting 960 μF. Selection for glutamine synthetase was done at 25 μM concentration of the L-methionine sulfoximine (Sigma) in glutamine-free Glasgow minimal medium supplemented with dialysed FBS, non-essential amino acids, glutamic acid, aspartic acid, sodium pyruvate, nucleosides, penicillin and streptomycin, essentially as has been described by Bebbington and Hentschel (1987). Colonies were picked 10 days after the selection, expanded and used for the second round of selection at 250 μM of L-methionine sulfoximine. This step was included to amplify the sequences coupled to the selection marker. Cell lines were expanded and tested for expression of E2 protein by immunoprecipitation with polyclonal rabbit antibodies against E2 protein (Ustav and Stenlund,1991) after labelling with [^{35}S]methionine using Translabel (ICN) and by a functional transient replication assay as described below.

E1 cell line CHO212. E1 protein expression vector pCGEag (Ustav and Stenlund, 1991) was linearized by *Xho*I. pBJ5GS was linearized, mixed with pCGEag at a 1:1 ratio, ligated into the concatemers and the cell line expressing E1 protein was generated in essentially the same way as for the E2-expressing cell line.

E1 and E2-expressing cell line CHO4.15. The E1 protein expression vector pE1-1×5 containing the glutamine synthetase minigene and E1

coding sequence was linearized with *Sal*I and pHSE2 was linearized with the *Xho*I restriction endonuclease. Linear plasmids were ligated into the concatemers at a ratio of 1:1. CHO-K1 cells were transfected by electroporation and selected as described above.

Transient replication assays

The assays were done as described earlier (Ustav and Stenlund, 1991) using the respective cell lines. For testing the cell lines in a functional assay for expression of the BPV-1 replication proteins, we used 50 ng of pUC/Alu (Ustav *et al.*, 1991) as the origin-containing plasmid and 250 ng of the E1 expression vector pE1-1×5 or pCGE2 to complement the E2 cell line CHO49 or the E1 cell line CHO212, respectively. All pNeo5′-based origin plasmids were tested for their ability to replicate in the CHO4.15 cell line by transfecting 100 ng of the plasmid DNA together with 50 μg of denatured carrier salmon sperm DNA into the CHO4.15 cells at 240 V by electroporation. Extrachromosomal DNA was extracted from the cells at 48 and 72 h post-transfection by alkaline lysis, as described earlier (Ustav and Stenlund, 1991). DNA was purified, digested with *Dpn*I and linearizing enzyme and analysed by Southern analysis. Specific probes for hybridization were made by random priming.

Stable replication

CHO4.15 cells were electroporated with 100 ng of origin-containing plasmid DNA in the presence of 50 μg of carrier DNA. Ninety-six hours after transfection, CHO4.15 cells were trypsinized and subjected to G418 selection at a concentration of 450 μg/ml. Colonies were pooled or single colonies were picked after 2 weeks, expanded and episomal or total DNA was analysed by Southern blotting.

Copy number measurement

Total DNA from established cell lines, containing replicating BPV-1 origin plasmids, was extracted and digested either with a plasmid single-cutter (*Hin*dIII) or a plasmid non-cutter (*Apa*I), followed by electrophoresis in 0.7% agarose–TAE gels. The copy number was measured by Southern blotting, using probe containing sequences from the BPV ori and the *neo* gene. Results were quantitated by comparing band intensity with a 2-fold dilution series of plasmid DNA using a PhosphorImager.

BrdU labelling and analysis of the replication mode

Cells were labelled with 35 μg/ml BrdU in MEM medium, containing 2′-deoxycytidine (20 μg/ml) using procedures described earlier (Yates and Guan, 1991). Episomal and chromosomal DNA was extracted at the times indicated in the Results section. CsCl solution was added to the DNA preparation to 1.74 g/ml and centrifuged for 48 h at 37000 r.p.m.. Twenty-four fractions were collected from each gradient, subjected to denaturation and neutralization, and slot-blotted onto nylon filters. Filters were hybridized with labelled BPV-1 or CHO genomic DNA probes. Radioactivity was counted with a PhosphorImager (Fuji).

Nuclear retention measurement by stable transformation assay

Forty-eight hours after transfection, the cells were trypsinized, counted and plated at three different dilutions. G418 selection was applied, and the selective medium was changed every 3 days. Colonies were counted after 10 days.

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References

Bebbington,C.R. and Hentschel,C.C.G. (1987) In Glover,D.M. (ed.), *DNA Cloning—A Practical Approach.* IRL Press, Oxford, Vol III, pp. 163–188.

Berg,L., Lusky,M., Stenlund,A. and Botchan,M.R. (1986) Repression of bovine papilloma virus replication is mediated by a virally encoded *trans*-factor. *Cell*, **46**, 753–762.

Blitz,I.L. and Laimins,L.A. (1991) The 68-kilodalton E1 protein of bovine papillomavirus is a DNA binding phoshoprotein which associates with the E2 transcriptional activator *in vitro*. *J. Virol.*, **65**, 649–656.

Bonne-Andrea,C., Santucci,S. and Clertant,P. (1995) Bovine papillomavirus E1 protein can, by itself, efficiently drive multiple rounds of DNA synthesis *in vitro*. *J. Virol.*, **69**, 3201–3205.

Botchan,M.R., Berg,L., Reynolds,J. and Lusky,M. (1986) The bovine papillomavirus replicon. In Evered,D. and Clark,S. (eds), *Papillomaviruses*. John Wiley & Sons, New York, pp. 53–67.

Chiang,C.-M., Ustav,M., Stenlund,A., Ho,T.F., Broker,T.R. and Chow,L.T. (1992) Viral E1 and E2 proteins support replication of homologous and heterologous papillomaviral origins. *Proc. Natl Acad. Sci. USA*, **89**, 5799–5803.

DePamphilis,M.L. (1993) Eukaryotic DNA replication: anatomy of the origin. *Annu. Rev. Biochem.*, **62**, 29–63.

DeVilliers,E.M. (1989) Heterogeneity of the human papillomavirus group. *J. Virol.*, **63**, 4898–4903.

Dvoretzky,I., Shober,R., Chattopadhyay,S.K. and Lowy,D.R. (1980) A quantitative *in vitro* focus assay for bovine papillomavirus. *Virology*, **103**, 369–375.

Gilbert,D.M. and Cohen,S.N. (1987) Bovine papilloma virus plasmids replicate randomly in mouse fibroblasts throughout S phase of the cell cycle. *Cell*, **50**, 59–68.

Hubert,W.G. and Lambert,P.F. (1993) The 23-kilodalton E1 phosphoprotein of bovine papillomavirus type 1 is nonessential for the stable plasmid replication in murine C127 cells. *J. Virol.*, **67**, 2932–2937.

Jacob,F., Brenner,S. and Cuzin,F. (1963) On the regulation of DNA replication in bacteria. *Cold Spring Harbor Symp. Quant. Biol.*, **28**, 329–348.

Jareborg,N., Alderborn,A. and Burnett,S. (1992) Identification and genetic definition of a bovine papillomavirus type 1 E7 protein and absence of a low copy-number phenotype exhibited by E5, E6 and E7 viral mutants. *J. Virol.*, **66**, 4957–4965.

Kirchmaier,A.L. and Sugden,B. (1995) Plasmid maintenance of derivatives of oriP of Epstein–Barr virus. *J. Virol.*, **69**, 1280–1283.

Krysan,P.J., Haase,S.B. and Calos,M.P. (1989) Isolation of human sequences that replicate autonomously in human cells. *Mol. Cell. Biol.*, **9**, 1026–1033.

Kuo,S.R., Liu,J.S., Broker, T.R. and Chow,L.T. (1994) Cell-free replication of the human papillomavirus DNA with homologous viral E1 and E2 proteins and human cell extracts. *J. Biol. Chem.*, **269**, 24058–24065.

Law,M.F., Lowy,D.R., Dvoretzky,I. and Howley,P.M. (1981) Mouse cells transformed by bovine papilloma virus contain only extrachromosomal viral DNA sequences. *Proc. Natl Acad. Sci. USA*, **78**, 2727–2731.

Li,R., Knight,J.D., Bream,G., Stenlund,A. and Botchan,M.R. (1989) Specific recognition nucleotides and their DNA context determine the affinity of E2 protein for 17 binding sites in the BPV-1 genome. *Genes Dev.*, **3**, 510–526.

Lu,J.Z., Sun,Y.N., Rose,R.C., Bonnez,W. and McCance,D.J. (1993) Two E2 binding sites (E2BS) alone or one E2BS plus an A/T-rich region are minimal requirements for the replication of the human papillomavirus type 11 origin. *J. Virol.*, **67**, 7131–7139.

Lusky,M. and Botchan,M.R. (1984) Characterization of the bovine papilloma virus plasmid maintenance sequence. *Cell*, **36**, 391–401.

Lusky,M. and Botchan,M.R. (1985) Genetic analysis of bovine papillomavirus plasmid type1 *trans*-acting replication factors. *J. Virol.*, **53**, 955–965.

Lusky,M. and Botchan,M.R. (1986) A bovine papillomavirus type 1 encoded modulator function is dispensable for transient viral replication but is required for the establishment of the stable plasmid state. *J. Virol.*, **60**, 729–742.

Middleton,T. and Sugden,B. (1994) Retention of plasmid DNA in mammalian cells is enhanced by binding of the Epstein–Barr virus replication protein EBNA-1. *J. Virol.*, **68**, 4067–4071.

Mohr,I.J., Clark,R., Sun,S., Androphy,E.J., MacPherson,P. and Botchan,M.R. (1990) Targeting the E1 replication protein to the papillomavirus origin of replication by complex formation with the E2 transactivator. *Science*, **250**, 1694–1699.

Müller,F., Seo,Y.S. and Hurwitz,J. (1994) Replication of bovine papillomavirus type 1 origin-containing DNA in crude extracts and with purified proteins. *J. Biol. Chem.*, **269**, 17086–17094.

Nallaseth,F.S. and DePamphilis,M. (1994) Papillomavirus contains *cis*-

acting sequences that can suppress but not regulate origins of DNA replication. *J. Virol.*, **68**, 3051–3064.

Nordström,K. (1990) Control of plasmid replication—how do DNA iterons set the replication frequency? *Cell*, **63**, 1121–1124.

Ravnan,J.-B., Gilbert,G.M., Ten Hagen,K.G. and Cohen,S.N. (1992) Random-choice replication of extrachromosomal bovine papillomavirus (BPV) molecules in heterogeneous clonally derived BPV-infected cell lines. *J. Virol.*, **66**, 6946–6952.

Roberts,J.M. and Weintraub,H. (1986) Negative control of DNA replication in composite SV40–bovine papillomavirus plasmids. *Cell*, **46**, 741–752.

Russell,J. and Botchan,M.R. (1995) *Cis*-acting components of human papillomavirus (HPV) DNA replication: linker substitution analysis of the HPV type 11 origin. *J. Virol.*, **69**, 651–660.

Schiller,J.T., Vass,W.C. and Lowy,R.D. (1984) Identification of a second transforming region in papillomavirus DNA. *Proc. Natl Acad. Sci. USA*, **81**, 7880–7884.

Spalholz,B.A., Lambert,P.F., Yee,C.L. and Howley,P.M. (1987) Bovine papillomavirus transcriptional regulation: localization of the E2-responsive elements of the long control region. *J. Virol.*, **61**, 2128–2137.

Stillman,B. (1994) Initiation of chromosomal DNA replication in eukaryotes. *J. Biol. Chem.*, **269**, 7047–7050.

Struhl,K., Stinchcomb,D.T., Scherer,S. and Davis,R.W. (1979) High-frequency transformation of yeast: autonomous replication of hybrid molecules. *Proc. Natl Acad. Sci. USA*, **76**, 1035–1039.

Svedrup,F. and Khan,S.A. (1995) Two E2 binding sites alone are sufficient to function as the minimal origin of replication of human papillomavirus type 18 DNA. *J. Virol.*, **69**, 1319–1323.

Szymanski,P. and Stenlund,A. (1991) Regulation of early gene expression from the bovine papillomavirus genome in transiently transfected C127 cells. *J. Virol.*, **65**, 5710–5720.

Tsurimoto,T., Melendy,T. and Stillman,B. (1990) Sequential initiation of lagging and leading strand synthesis by two different polymerase complexes at the SV40 DNA replication origin. *Nature*, **346**, 534–539.

Ustav,E., Ustav,M., Szymanski,P. and Stenlund,A. (1993) The bovine papillomavirus origin of replication requires a binding site for the E2 transcriptional activator. *Proc. Natl Acad. Sci. USA*, **90**, 898–902.

Ustav,M. and Stenlund,M. (1991) Transient replication of BPV-1 requires two viral polypeptides encoded by the E1 and E2 open reading frames. *EMBO J.*, **10**, 449–457.

Ustav,M., Ustav,E., Szymanski,P. and Stenlund,A. (1991) Identification of the origin of replication of bovine papillomavirus and characterisation of the viral origin recognition factor E1. *EMBO J.*, **10**, 4321–4329.

Vande Pol,S.B. and Howley,P.M. (1992) A bovine papillomavirus constitutive enhancer is essential for viral transformation, DNA replication and the maintenance of latency. *J. Virol.*, **66**, 2346–2358.

Wahl,G.M. (1989) The importance of circular DNA in mammalian gene amplification. *Cancer Res.*, **49**, 1333–1340.

Weinberg,D.H., Collins,K.L., Simancek,P., Russo,A., Wold,M.S., Virshup,D.M. and Kelly,T.J. (1990) Reconstitition of simian virus 40 DNA replication with purified proteins. *Proc. Natl Acad. Sci. USA*, **87**, 8692–8696.

Windle,B. and Wahl,G.M. (1992) Molecular dissection of mammalian gene amplification: new insights revealed by analyses of very early events. *Mutat. Res.*, **276**, 199–224.

Windle,B., Draper,B., Yin,Y., O'Gorman,S. and Wahl,G.M. (1991) A central role for chromosome breakage in gene amplification, deletion formation and amplicon integration. *Genes Dev.*, **5**, 160–174.

Yang,L., Li,R., Mohr,I.J., Clarck,R. and Botchan,M.R. (1991) Activation of BPV-1 replication *in vitro* by the transcription factor E2. *Nature*, **353**, 628–632.

Yates,J.L. and Guan,N. (1991) Epstein–Barr virus-derived plasmids replicate only once per cell-cycle and are not amplified after entry into cells. *J. Virol.*, **65**, 483–488.

Yates,J., Warren.,N., Reisman,D. and Sugden,B. (1984) A *cis*-acting element from the Epstein–Barr viral genome that permits stable replication of recombinant plasmids in latently infected cells. *Proc. Natl Acad. Sci. USA*, **81**, 3806–3810.

Yates,J.L., Warren,N. and Sugden,B. (1985) Stable replication of plasmids derived from Epstein–Barr virus in various mammalian cells. *Nature*, **313**, 812–815.

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